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Extraordinarily few organisms of a live recombinant BCG vaccine against tuberculosis induce maximal cell-mediated and protective immunity

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Abstract

In previous studies, we have described a live recombinant BCG vaccine (rBCG30) overexpressing the 30 kDa major secretory protein of *Mycobacterium tuberculosis* that induces greater protective immunity against tuberculosis than the current vaccine in the demanding guinea pig model of pulmonary tuberculosis. In this study, we have investigated the impact of vaccine dose on the development of cell-mediated and protective immunity in the guinea pig model. We found that the protective efficacy against *M. tuberculosis* aerosol challenge of both BCG and rBCG30 was essentially dose-independent over a dose range of $10^1$ to $10^6$ live organisms. As previously observed, rBCG30 was more potent, reducing colony-forming units (CFU) below the level observed in animals immunized with the parental BCG vaccine by 0.7 logs in the lungs and 1.0 logs in the spleen ($P<0.0001$). To gain a better understanding of the influence of dose on bacterial clearance and immunity, we assessed animals immunized with $10^1$, $10^3$, or $10^6$ CFU of rBCG30. The higher the dose, the higher the peak CFU level achieved in animal organs. However, whereas humoral immune responses to the 30 kDa protein reflected the disparate CFU levels, cell-mediated immune responses did not; high and low doses of rBCG30 ultimately induced comparable peak lymphocyte proliferative responses and cutaneous delayed-type hypersensitivity responses to the 30 kDa protein. We estimate that the amount of the 30 kDa protein required to induce a strong cell-mediated immune response when delivered via 10 rBCG30 organisms is about 9 orders of magnitude less than that required when the protein is delivered in a conventional protein/adjuvant vaccine. This study demonstrates that a very low inoculum of rBCG30 organisms has the capacity to induce strong protective immunity against tuberculosis and that rBCG30 is an extremely potent delivery system for mycobacterial antigens.
1. Introduction

Despite advances in many areas of medicine, tuberculosis remains a leading cause of death and disability worldwide. A better vaccine than the current widely used vaccine, bacille Calmette-Guérin or BCG, holds the greatest hope for making a major impact on disease incidence.

We have previously described a new vaccine against tuberculosis that is more potent than BCG in the guinea pig model of pulmonary tuberculosis, an animal model noteworthy for its high susceptibility to infection with *M. tuberculosis*, the primary causative agent of tuberculosis in humans, and for its clinical, pathologic, and immunologic relevance to human tuberculosis (1, 2). The new vaccine, known as rBCG30, is a recombinant BCG that overexpresses the *M. tuberculosis* 30 kDa major secretory protein. Guinea pigs immunized with rBCG30 and then challenged with highly virulent *M. tuberculosis* by aerosol so as to mimic the natural route of infection, have significantly less organ pathology, significantly fewer organisms in their lungs and spleen, and significantly greater survival than guinea pigs immunized with the parental strain of BCG (1, 2).

rBCG30 is currently being evaluated for safety and efficacy in humans. A relevant issue is the dose of vaccine to be administered. In our previous animal experiments, we used a dose of $10^3$ colony-forming units (CFU) of vaccine intradermally, a dose in the range of BCG doses typically used in guinea pigs (3). In humans, doses of intradermally administered BCG are typically higher, in the range of $10^5$ to $10^6$ CFU (4). However, the relationship of BCG dose to efficacy in humans, if any, is unknown, a deficiency in our knowledge that is difficult to correct given the absence of known correlates of protective immunity against tuberculosis in humans.

Previous studies have provided grounds for postulating that the protective immunity induced by BCG may vary directly with dose, vary inversely with dose, or be dose-independent. In support of a direct dependence, some studies involving immunization of animals with various strains of BCG followed by challenge with *M. tuberculosis* or the highly related mycobacterial pathogen
Mycobacterium bovis have found that higher doses of the vaccine resulted in increased survival of the animals and/or decreased growth of M. tuberculosis in animal organs (3, 5-11). In some of these studies, however, the decreased efficacy of low doses may have resulted from an absence of viable organisms in the vaccine inoculum (7, 8). In others, the protective immunity induced by low doses may have been not so much diminished as delayed (6, 9, 12). In support of an inverse dependence of protective immunity on dose, one study in deer immunized twice with BCG and then challenged with M. bovis found that a higher dose was inferior to a lower dose (13). Another study observed that a low dose of BCG vaccine favors a TH1 type of immune response vs. a TH2 type of response in Balb/c mice (14); TH1 type responses are considered to favor enhanced protective immunity against intracellular parasites, most convincingly in the case of Leishmania. Finally, several studies of BCG vaccine indicate an absent effect of dose on protective immunity, particularly when the immunization-challenge interval is greater than 7 weeks (6, 12, 15-21). Whatever the relationship between dose and efficacy of wild-type BCG, whether a more potent recombinant BCG vaccine would exhibit the same dose-efficacy relationship was not known before the present study.

In this study, we have examined the impact of the dose of rBCG30 on efficacy in the guinea pig model. We shall show that the development of cell-mediated and protective immunity is essentially dose-independent over a 5 log range - only the rapidity with which cell-mediated immunity develops is dose-dependent. Remarkably, extraordinarily few organisms (≤10) are sufficient to induce maximal levels of cell-mediated and protective immunity.
2. Materials and methods

2.1. Bacterial strains

The vaccines tested were the parental *Mycobacterium bovis* BCG Tice (BCG) and a recombinant BCG Tice (rBCG30) overexpressing the 30 kDa major secretory protein, a mycolyl transferase also known as Antigen 85B (22). The challenge strain was *Mycobacterium tuberculosis* Erdman strain (ATCC 35801).

2.2. Effect of dose on protective immunity

Specific-pathogen free 250- to 300-g outbred male Hartley strain guinea pigs from Charles Rivers Breeding Laboratories (Wilmington, MA) were injected intradermally with parental BCG or rBCG30, prepared as previously described (1, 2), in doses ranging in 10-fold increments from $10^1$ to $10^6$ CFU (n = 9 or 18 animals per group). The preparation of the vaccines entailed washing the bacteria by centrifugation, enumerating them in a Petroff-Hausser chamber, and resuspending them in PBS; such bacteria had a very high (>90%) CFU:particle ratio. Control animals were sham-immunized with buffer (phosphate-buffered saline) only (n = 9 animals per group). Ten weeks later, the guinea pigs were challenged with an aerosol generated from a 7.5-mL single-cell suspension containing a total of $7.5 \times 10^4$ CFU of *M. tuberculosis* using a Collison 6-jet nebulizer (BGI Instruments, Waltham, MA) at a pressure of 20 psi; this dose delivered ~10 live bacteria to the lungs of each animal, based on counting primary lesions in the lungs of animals euthanized three weeks after exposure to this dose. Afterwards, guinea pigs were individually housed in stainless steel cages contained within a laminar flow biohazard safety enclosure and allowed free access to standard laboratory food and water. The animals were observed for illness and weighed weekly for 10 weeks and then euthanized. The lungs, spleen, and liver of each animal were removed aseptically and inspected.
immediately for pathology, and the right lung and spleen were cultured for CFU of *M. tuberculosis*.

2.3. Effect of dose on dissemination and clearance of rBCG30 and development of lymphocyte proliferative responses to *M. tuberculosis* antigens

Guinea pigs were immunized intradermally as above with $10^1$, $10^3$, or $10^6$ CFU of rBCG30. At various intervals thereafter, the animals were euthanized and the right lung and three-quarters of the spleen assayed for CFU of rBCG30 as described (2). CFU counts for the spleen were corrected for the whole organ based on the weight of the whole spleen and the portion assayed. Serial 10-fold dilutions of each lung and spleen homogenate were plated; in addition, undiluted homogenate equivalent to one-half of the entire organ was plated on 20 agar plates to allow accurate measurement of the bacterial load where very low numbers of bacteria were present in an organ. Where no bacteria were detected, CFU counts per organ were scored as 1 CFU (0.0 log).

The quarter of the spleen not assayed for CFU was utilized for lymphocyte proliferation studies. Splenic lymphocytes were purified as described (22) and incubated at a final concentration of $10^7$/ml in RPMI1640 containing 12.5 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), polymyxin B sulfate (100 Units/ml), and 10% fetal calf serum (Gibco) with PPD (10 μg/ml) or with 100, 10, or 1 μg/ml of purified recombinant *M. tuberculosis* 30 kDa major secretory protein (r30) in a total volume of 100 μl in microtest wells (96-well round-bottom tissue culture plate; #3007; Falcon Labware, Oxnard, CA) for 2 days at 37°C in 5%CO$_2$-95%air and 100% humidity. As negative and positive controls, lymphocytes were incubated with buffer only (RPMI) or with concanavalin A (15μg/ml). Subsequently, [3H]thymidine incorporation was determined essentially as described (22).
2.4. Effect of dose on antibody responses

Blood was obtained from the animals described above immediately before they were euthanized, and the serum was assayed for antibody titer to r30 by ELISA, using Costar (Corning, NY) 96-well EIA/RIA High Binding Plates, r30 at 1 μg/well, guinea pig serum diluted 1:250 to 1:1,024,000, alkaline phosphatase-conjugated goat anti-guinea pig IgG (Sigma, St. Louis, MO) at a dilution of 1:1,000, and an Alkaline Phosphatase Substrate Kit (BioRad, Hercules, CA).
3. Results

3.1 The protective immunity induced by both rBCG30 and BCG vaccines in guinea pigs is dose-independent

To determine the impact of vaccine dose on protective immunity, we immunized guinea pigs intradermally with $10^1$ to $10^6$ CFU of rBCG30 or BCG, challenged the animals with aerosolized *M. tuberculosis* 10 weeks later, and monitored the subsequent course of infection. As an objective indicator of illness, we assessed weight loss, a major physical sign of tuberculosis in humans and a hallmark of the disease in the guinea pig model of pulmonary tuberculosis. All groups of animals immunized with rBCG30, regardless of dose, gained weight normally after challenge. There was no significant difference in weight gain among these 12 groups of infected animals or between groups of infected animals and uninfected controls. In contrast, after gaining 50 g during the first two weeks after challenge, sham-immunized animals lost this much weight over the subsequent 8 weeks, such that by the end of the 10-week observation period, the mean weight of the animals in the sham-immunized group was nearly the same as it was just before challenge and significantly less than that of BCG or rBCG30 immunized animals [$P<0.0001$ by parametric analysis of variance methods (ANOVA) and $P\leq0.0004$ by nonparametric Kruskal-Wallis methods (K-W)(data not shown)].

To assess the impact of vaccine dose on growth of *M. tuberculosis* in the tissues of challenged guinea pigs, we euthanized the animals at 10 weeks and assayed CFU in the lung and spleen (Fig. 1). Sham-immunized animals (n=9) had the highest bacterial load in these organs - an average of $6.1\pm0.3$ log CFU in the lungs and $5.7\pm0.3$ log CFU in the spleens. Animals immunized with BCG or rBCG30, regardless of dose, had significantly fewer CFU in the lungs and spleen than sham-immunized animals. Among animals immunized with BCG, the number of CFU in the lungs and spleen was similar at all doses except for the lowest dose, where animals had somewhat greater mean CFU in the lungs;
however, the differences in CFU between this group and each of the other BCG-immunized groups were not statistically significant. Combining all doses, BCG-immunized animals (n=63) had a mean of 4.7±0.1 log CFU in the lungs and 3.7±0.1 log CFU in the spleens, which was 1.4 log fewer CFU in the lungs and 2.0 log fewer CFU in the spleens than sham-immunized animals. These differences were highly significant (P<0.0001 in the lungs and spleen by both ANOVA and K-W).

Animals immunized with rBCG30 had significantly fewer CFU in the lung and spleen than both sham-immunized and BCG-immunized animals. Combining all doses, rBCG30-immunized animals (n=63) averaged 4.0±0.1 log CFU in their lungs and 2.7±0.1 log CFU in their spleens, which was 0.7 log fewer CFU in the lungs and 1.0 log fewer CFU in the spleens than BCG-immunized animals, differences that statistically were highly significant (P< 0.0001 in the lung and spleen by ANOVA and K-W). Again, there was no significant relationship between dose and efficacy.

3.2 Vaccine dissemination but not the development of cell-mediated immunity is dose-dependent

We hypothesized that the dose-independent effect of the vaccine reflected the fact that the high doses and low doses of the vaccine multiply to comparable levels in animal tissues and induce a comparable immune response. To test this hypothesis, we immunized animals intradermally with the highest dose (10^6 CFU) and the lowest dose (10^1 CFU) of rBCG30, and assessed dissemination by euthanizing animals at various times after immunization and assaying CFU of rBCG30 in the lungs and spleen. In a previous study of vaccine dissemination and clearance, in which guinea pigs were immunized intradermally with 10^3 CFU of rBCG30, we found that the vaccine strain multiplied in animal tissues to a peak level three weeks after immunization of 2.9±0.1 logs (Mean ± SE) in the lungs and 3.2±0.2 logs in the spleen, after which the levels rapidly declined to low levels (<10^1 CFU) by 10 weeks (2). These low levels persisted for at least 26
weeks after immunization (2). In the present study, we found that in animals immunized with the $10^6$ CFU dose of rBCG30, the vaccine strain similarly reached peak levels in animal organs 3 weeks after immunization (Fig. 2, Experiment 1); the levels that were reached -- 3.7±0.1 logs in the lung and 4.4±0.3 logs in the spleen -- were higher (~ 1 log CFU) than with the $10^3$ CFU dose in the previous study. Surprisingly, and contrary to our expectations, in animals immunized with the $10^1$ CFU dose, the vaccine strain failed to reach high levels in the lung and spleen; indeed CFU were virtually undetectable in these organs. Hence, in contrast to protective immunity, the level attained by the vaccine strain in the lungs and spleen was proportional to dose. These results were confirmed in a second experiment in which immunization doses of $10^1$, $10^3$, and $10^6$ CFU of rBCG30 were tested; again, the extent of bacterial dissemination was dose-dependent (Fig. 2, Experiment 2).

Cell-mediated immunity is central to host defense against *M. tuberculosis*. To determine the relationship of dose to cell-mediated immunity, we assayed the development of lymphocyte proliferative responses to *M. tuberculosis* antigens in splenic lymphocytes of animals immunized with $10^1$ or $10^6$ CFU of rBCG30 (Experiment 1) or with $10^1$, $10^3$, or $10^6$ CFU of rBCG30 (Experiment 2) -- the same animals in which CFU levels in the lungs and spleens were assayed above (Fig. 3). As described in Materials and Methods, one portion (three-quarters) of each animal's spleen was assayed for CFU and the remaining portion served as a source of lymphocytes. The *M. tuberculosis* antigens studied were the 30 kDa major secretory protein – the protein overproduced by the vaccine – and PPD, a crude extract of *M. tuberculosis* known as tuberculin that is used for skin-testing of humans for exposure to *M. tuberculosis*. In Experiment 1, in animals immunized with $10^6$ CFU rBCG30, lymphocyte proliferative responses to the *M. tuberculosis* antigens increased rapidly after immunization. Responses to both the 30 kDa protein and PPD were significantly greater than background even one week after immunization. The lymphocyte proliferative responses rapidly increased during the subsequent several weeks until they reached a plateau at 3-4 weeks. In animals immunized with $10^1$ CFU rBCG30, the lymphocyte
proliferative responses developed later. Significant responses did not develop until 3 weeks after immunization. Responses subsequently increased until by 6-8 weeks they reached levels comparable to that observed in animals immunized with $10^6$ CFU rBCG30 at 3-4 weeks. Hence, the development of strong lymphocyte proliferative responses to *M. tuberculosis* antigens in animals immunized with $10^1$ CFU of rBCG30 lagged by 3-4 weeks the development of such responses in animals immunized with $10^6$ CFU. Similarly, in Experiment 2, the rate at which animals developed lymphocyte proliferative responses significantly above background and the rate at which they reached high levels was dose-dependent. In this experiment, animals immunized with the highest dose achieved a higher peak level, at 3 weeks after immunization, than animals immunized with the lower two doses, but within a few weeks, the responses of all three groups were roughly comparable. Interestingly, animals immunized with the lowest dose showed a spike in lymphocyte proliferative activity at 15 weeks. Thus, in both experiments, the rate of development of lymphocyte proliferative responses was proportional to dose, but all animals eventually achieved a comparably vigorous lymphocyte proliferative response.

In a separate group of animals, we assayed the development of cutaneous delayed-type hypersensitivity (c-DTH) responses to *M. tuberculosis* antigens in animals immunized with $10^1$ or $10^6$ CFU of rBCG30 (Fig. 4). Animals immunized with $10^6$ CFU rBCG30 developed a very vigorous c-DTH response by 3 weeks after immunization that averaged 26.7±1.9 mm induration. In contrast, animals immunized with $10^1$ CFU rBCG30 showed a negligible c-DTH response at 3 weeks. By 6 weeks, the $10^1$ CFU-immunized animals had developed a significant c-DTH response averaging 10.1±3.3 mm induration, but a response still less than half that of the $10^6$ CFU-immunized animals (24.2±1.0 mm induration) at that time point. By 9 weeks, the mean c-DTH responses of the high and low dose groups were comparable, and they remained so until the experiment was terminated at 15 weeks. Thus, as with lymphocyte proliferative responses to *M. tuberculosis* antigens, c-DTH responses developed more slowly.
in animals immunized with a low vaccine dose, but eventually reached a level comparable to that of animals immunized with a high vaccine dose.

3.3 The humoral immune response, in contrast to the cell-mediated immune response, is dose-dependent

In Experiment 1, antibody responses were assayed in the same animals studied for lymphocyte proliferative responses to M. tuberculosis antigens. Animals immunized with $10^6$ CFU of rBCG30 rapidly developed antibody to r30 (Fig. 5); significant responses above background were evident even one week after immunization in 2 of the 3 animals tested [unimmunized guinea pigs have antibody titers to r30 of $\leq 125$ (data not shown)]. By three weeks after immunization with $10^6$ CFU of rBCG30, high antibody titers had developed in all animals, and these titers remained high thereafter until the experiment was terminated at 10 weeks. In contrast, animals immunized with $10^1$ CFU of rBCG30 did not develop high antibody titers (Fig. 5). Most animals never developed antibody titers above background. At 4, 6, 8, and 10 weeks, one animal per group developed a modest titer above background. Hence, the antibody titer, in contrast to the cell-mediated immune response, reflected the level of bacterial dissemination.
4. Discussion

Our study demonstrates that the efficacy of both the recombinant rBCG30 vaccine and the parental BCG vaccine in the guinea pig model of pulmonary tuberculosis is essentially dose-independent. These results are consistent with the preponderance of earlier studies of BCG vaccine indicating little or no effect of dose on efficacy in a variety of animal models of tuberculosis including mice, voles, guinea pigs, and cattle, especially when the immunization-challenge interval was greater than seven weeks. That efficacy is dose-independent across a variety of animal species, some more and some less susceptible to tuberculosis than humans, suggests that the efficacy of BCG in humans may also be dose-independent. By extension and in view of the demonstrated dose-independence of rBCG30 efficacy in guinea pigs in this study, the efficacy of rBCG30 in humans may also be dose-independent.

Remarkably, the lowest dose of live vaccine administered - $10^1$ CFU intradermally - barely multiplied in animal organs. Only a few organisms were detectable in the lungs and spleens - two organs that are major sites of vaccine dissemination. Nevertheless, this dose was capable of inducing maximal cell-mediated and protective immunity.

Although the cell-mediated immune response developed more slowly in animals immunized with the $10^1$ CFU dose compared with animals immunized with higher doses, the response was evidently sufficient to check the growth of the vaccine, as the numbers of rBCG30 in the lungs and spleens of immunized animals remained very low throughout the post-vaccination period. Hence, whether rBCG30 reached high or low levels in animal organs, the immune response was sufficient to contain its growth.

In sharp contrast to the cell-mediated immune response, the humoral immune response to rBCG30 was strictly dose-dependent. Few animals immunized with the low dose ($10^1$ CFU) developed antibody titers above background and even then the titers were modest. In contrast, animals immunized with the high dose ($10^6$ CFU) developed very high antibody titers.
The dichotomy between the dose-independence of the cell-mediated immune response and the dose-dependence of the humoral immune response in this study was striking. With respect to the T-cell response, the results suggest that extraordinary few MHC-peptide complexes on antigen presenting cells in animals immunized with the $10^1$ CFU dose of rBCG30 were able to stimulate an extremely vigorous and protective T-cell proliferative response. With respect to the B-cell response, the poor antibody development of the animals immunized with the $10^1$ CFU dose of rBCG30, which was rapidly cleared from animal organs, may reflect the dependence of B-cell maturation on the continued presence of antigen. In contrast, the high antibody titers of the animals immunized with the high dose of rBCG30, who demonstrated high levels of organisms in their organs for several weeks after immunization, suggest that sufficient bacteria and r30 protein were available for several weeks after immunization to develop and sustain a vigorous B-cell response.

rBCG30 evidently is an incredibly powerful adjuvant delivery system for r30 and other mycobacterial antigens as evidenced by the strong lymphocyte proliferative responses to r30 and PPD and the strong cutaneous DTH responses to r30 after intradermal inoculation of only 10 CFU. These strong cell-mediated immune responses developed despite there being surprisingly limited if any multiplication of the vaccine in the lung and spleen, two major sites of vaccine dissemination in the guinea pig that appear to reflect bacterial dissemination generally in this host (2); for example, in a previous study, the extent of dissemination and the kinetics of clearance of BCG and rBCG30 in the guinea pig lung and spleen mirrored that in the regional lymph nodes (2). The amount of antigen released by the vaccine in vivo is difficult to estimate based on currently available data. In a three-week broth culture of rBCG30, by which time the organism reaches late log phase, each bacterium releases on average approximately 5 femtograms of r30. Based on clearance studies of rBCG30 in this and previous studies, rBCG30 is present in vivo at numbers approximating the original intradermal dose for several weeks before being cleared to low levels well below the intradermal dose. If as a first approximation, we assume that the
same amount of r30 is produced per bacterium in vivo as in vitro, then the 10 CFU dose of rBCG30 would release about 50 femtograms of r30. This is approximately 9 orders of magnitude less than the amount of r30 protein – 10 to 100 μg – required to induce a strong cell-mediated immune response when delivered in a conventional protein/adjuvant vaccine; moreover, several doses of the conventional vaccine are required (1, 24). A similar amount of a protective protein antigen is required in a conventional protein/adjuvant vaccine to induce a strong cell-mediated and protective immune response against Legionella pneumophila in guinea pigs (25, 26). Even if our crude estimate of 50 femtograms is short by as much as 10- or 100-fold, this amount of r30 is still many orders of magnitude less than the level of antigen required in conventional vaccines to induce strong immunologic responses and protective immunity (1, 24-26).

The M. tuberculosis 30 kDa protein has proven to be a potent immunoprotective antigen when delivered via rBCG30. There are several possible explanations for its high potency. First, the original rationale for selecting this antigen was the Extracellular Protein Hypothesis for vaccines against intracellular parasites, which holds that proteins secreted or otherwise released by intracellular pathogens are key immunoprotective molecules because their processing and presentation on the host cell surface as part of MHC-peptide complexes allows the immune system to recognize an infected host cell and exert an antimicrobial effect against it (1, 2, 23-26). In this regard, the 30 kDa protein is the major protein secreted by M. tuberculosis in broth culture (24) and among the most abundantly expressed proteins of all types produced by M. tuberculosis within human macrophages (27). Second, the 30 kDa protein has a large number of immunodominant T-cell epitopes in both guinea pigs and humans (28, 29), allowing it to induce immunoprotection across disparate MHC types in outbred populations. Third, the 30 kDa protein, a mycolyl transferase (22), synthesizes the key cell wall component trehalose dimycolate, potentially enhancing rBCG30's cell wall integrity, resistance to degradation, and immunogenicity. Consistent with this idea, rBCG30 is slightly
more resistant to the antibiotic isoniazid, an inhibitor of mycolic acid synthesis (2).

In conclusion, the efficacy of rBCG30, the first vaccine demonstrated more potent than BCG in preclinical studies and now in human trials, is dose-independent over a 5-log range - $10^1$ to $10^6$ CFU administered intradermally - in the highly relevant guinea pig model of pulmonary tuberculosis. Low doses of the vaccine do not reach as high a level in animal organs as high doses, but ultimately induce as great a cell-mediated immune response to the 30 kDa protein and other mycobacterial antigens as high doses. High doses reach higher levels in animal organs, and probably as a consequence, induce a greater humoral immune response to the vaccine-encoded *M. tuberculosis* 30 kDa protein, but this does not result in greater immunoprotection. Hence, vaccine efficacy reflects the cell-mediated but not the humoral immune response, consistent with the critical role of cell-mediated immunity in host defense against *M. tuberculosis*. Remarkably, despite very little bacterial dissemination, an extraordinarily low number of rBCG30 (10 CFU) is able to induce a highly potent cell-mediated and protective immune response.
Acknowledgments

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References


Figure legends

**Fig. 1.** Protective immunity induced by immunization with $10^1$-$10^6$ CFU of BCG or rBCG30. Guinea pigs were immunized intradermally with $10^1$-$10^6$ CFU of BCG or rBCG30, and ten weeks later, the animals were challenged by aerosol with virulent *M. tuberculosis*. Ten weeks after challenge, the guinea pigs were euthanized and CFU in the right lung and spleen were assayed. Data are the mean ± SE for all animals in a group.

**Fig. 2.** Dissemination and clearance of $10^1$, $10^3$, or $10^6$ intradermally administered rBCG30 in animal organs. Guinea pigs were immunized intradermally with $10^1$ or $10^6$ CFU of rBCG30 (Experiment 1) or with $10^1$, $10^3$, or $10^6$ CFU of rBCG30 (Experiment 2) and euthanized 1 to 26 weeks later as indicated (n=3/group/timepoint). CFU were assayed in the right lung and spleen. Data are the mean CFU ± SE. Where 0 CFU were present in the homogenate of one-half of the entire organ, the CFU count was designated as 1 CFU or 0.0 log.

**Fig. 3.** Lymphocyte proliferation to *M. tuberculosis* antigens after high and low dose immunization. Splenic lymphocytes were isolated from the same animals studied in Fig. 2 and proliferative responses were assayed to r30 at doses of 10, 1, and 0.1 μg/well (100, 10, and 1 μg/ml) and to PPD at 1 μg/well (10 μg/ml). RPMI medium served as a negative control. Only the responses to the highest dose of r30 are shown; responses to lower doses of r30 were similar but slightly lower in a dose-dependent fashion. Data are the mean CPM ± SE per well.

**Fig. 4.** Cutaneous DTH to r30 after high and low dose immunization. A separate group of guinea pigs (n=3/group) was assayed for cutaneous DTH in response to r30 at 3, 6, 9, 12, and 15 weeks after immunization with $10^1$ or $10^6$ CFU of rBCG30. Data are mean induration (mm ± SE).
**Fig. 5.** Antibody titer to r30 after high and low dose immunization. Serum was obtained from the same Experiment 1 animals described in Fig. 2 and assayed for antibody to r30 by ELISA. Data are the reciprocal antibody titer for each individual animal (closed circles) and the geometric mean titer (bar) for each group. For statistical purposes, titers of \( \leq 125 \) were scored as 125.
Figure 1
Figure 2
Experiment 1

$10^1$ rBCG30

$10^6$ rBCG30

Lung

Spleen

Mean Log CFU ± SE

Week

Experiment 2

$10^1$ rBCG30

$10^3$ rBCG30

$10^6$ rBCG30

Lung

Spleen

Mean Log CFU ± SE

Week
Figure 3
Figure 4
Figure 5